

Antitumor effects of an engineered and energized fusion protein consisting of an anti-CD20 scFv fragment and lidamycin

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Antibody-based fusion proteins are the next generation of antibody therapies for cancer and other diseases. CD20 antigen, which is overexpressed on cell membranes in nearly 95% of cases of B-cell Non-Hodgkin's Lymphoma, is an attractive target for the therapy of B-lymphoid malignancies. Lidamycin (LDM) is a potent enediyne-containing antitumor antibiotic that now has entered phase II clinical trials. In this study, we prepared an engineered fusion protein, scFv-LDP, consisting of an anti-CD20 scFv fragment and the apoprotein LDP of LDM using DNA recombination. After purification and refolding, scFv-LDP was found to bind specifically to CD20-positive lymphoma cells using ELISA and indirect immunofluorescent cytochemical staining assays. The energized fusion protein scFv-LDP-AE was obtained using molecular reconstitution of the active chromophore AE of LDM and scFv-LDP. MTT assay revealed potent cytotoxicity of scFv-LDP-AE to CD20-positive Raji and Daudi cells, with IC₅₀ values of 1.21×10^{-11} and 6.24×10^{-11} mol L⁻¹, respectively. An *in vivo* subcutaneous xenograft model of CD20-positive B cell lymphoma in BALB/c (nu/nu) mice was also utilized. Drugs were given intravenously on day 14 and 21 after tumor transplantation. In terms of maximal tolerated doses, scFv-LDP-AE at 0.3 mg kg⁻¹ suppressed tumor growth by 79.3%, and LDM at 0.05 mg kg⁻¹ by 68.6% ($P < 0.05$). Results suggested scFv-LDP-AE could be a potential candidate for tumor-targeting therapy.

lymphoma, CD20, lidamycin, scFv, energized fusion protein

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Non-Hodgkin's Lymphoma (NHL) is a group of malignant diseases originating from the lymphatic system [1], and is the fifth most common malignancy in the world. Although conventional chemotherapy and radiotherapy with NHL treatment is effective, these treatment methods may also kill some normal cells because of their low selectivity, leading to obvious side effects. Therefore targeting therapy is becoming more and more important for increasing the effectiveness of cancer treatment. Most NHL originates from B lymphocytes, and CD20 antigen is overexpressed on the

surface of over 95% of NHL cells. CD20 is expressed on pre-B cells, immature B cells, mature B cells, and resting activated B cells, but is absent from plasma cells, pluripotent stem cells, and other cell types. By contrast, almost no free CD20 is found in serum. Therefore, CD20 is an ideal target antigen for monoclonal antibody therapy in B-cell NHL [2]. The US FDA has approved three monoclonal antibodies against CD20 on lymphoma cells, including Rituximab, Zevalin and Bexxar [3–5]. In fact the worldwide sales of Rituximab were approximately \$5.6 billion in 2009. Recently anti-CD20 antibodies have attracted wide attention because of their new therapeutic uses in a variety of auto-

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immune diseases [6] (e.g. rheumatoid arthritis [7], systemic lupus erythematosus [8,9] and pemphigus [10]). HI47 (IgG3), a murine monoclonal antibody against CD20, was developed by the Institute of Hematology, Chinese Academy of Medical Sciences in 1990, and was used in the present study. This antibody was named CD20+X at the 4th International Conference on Human Leukocyte Differentiation Antigens [11].

A single-chain variable fragment (scFv) is a fusion protein of the variable regions of the heavy (VH) and light chains (VL) in immunoglobulins, connected with a flexible linker peptide usually composed of (Gly₄Ser)₃ [12]. With a size of approximately 25 kD, scFv has many advantages over intact antibodies. scFv has no Fc-receptor binding activation, shows better penetration into solid tumors, has much lower immunogenicity, and can be produced in prokaryotes. These characteristics render scFv an attractive alternative to intact antibodies as a targeting drug carrier for therapeutic applications.

Lidamycin (LDM), also called C-1027, is produced by a *Streptomyces* strain isolated from a soil sample collected in China. As a member of the enediyne-containing antitumor antibiotics family, LDM is one of the most potent antitumor macromolecular peptide antibiotics ever reported [13]. In terms of IC₅₀ values, the cytotoxicity of LDM was 10000-fold more potent than that of doxorubicin [14,15]. LDM contains an enediyne chromophore (843 Da), responsible for the extremely potent bioactivity, and a noncovalently bound apoprotein LDP (10500 Da), which forms a hydrophobic pocket for protecting the chromophore. The apoprotein and chromophore can be dissociated and reconstituted, and the biological activity of the rebuilt molecule is comparable to that of natural LDM [16–18]. *In vivo* experiments demonstrated the marked growth inhibition LDM exerted on different transplantable tumors in mice [19–21]. LDM can cause site-specific cleavage of DNA, induce apoptosis in cancer cells, and inhibit tumor metastasis as well as angiogenesis. All of these responses showed that LDM could serve as a promising ‘warhead’ compound for preparing antibody-based therapeutics [22]. LDM has now entered phase II clinical trials.

In the present study we utilized the properties of LDM, which can be dissociated and reconstituted, to prepare an antibody-based fusion protein, anti-CD20 scFv-LDP-AE. Within this fusion protein, the scFv of antibody HI47 served as a vehicle and LDM as a “warhead”. The antitumor effects of scFv-LDP-AE were subsequently studied.

1 Materials and methods

1.1 Materials

1.1.1 Plasmids and strains

Both *E. coli* 16C9 and the expression vector pAYZ-scFv

carrying the gene for the scFv (anti-CD20) were separately preserved and constructed in the Institute of Hematology, CAMS & PUMC (Tianjin, China). The PGEM-T-LDP vector carrying the gene for apoprotein LDP of LDM was constructed in our laboratory. The pMD 18-T vector and *E. coli* DH5 α were purchased from Takara (Dalian, China) and BioDev-Tech (Beijing, China), respectively.

1.1.2 Cells

The B-cell lymphoma line, Raji and Daudi cells, were cultured in RPMI-1640 supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO₂ atmosphere.

1.1.3 Drugs and reagents

Restriction endonucleases and T4 DNA ligases were purchased from Takara (Dalian, China); mouse anti-His-Tag antibody were from Novagen (Darmstadt, Germany); FITC-labeled goat anti-mouse IgG was from Zhongshan Golden Bridge Biotechnology (Beijing, China); horseradish peroxidase-conjugated goat anti-mouse IgG and chemiluminescence detection reagent were from Santa Cruz (California, USA); PVDF membranes, Centriplus YM or Ultrafree-MC filter units were from Millipore (USA); Ni-NTA resin affinity chromatography column and Sephadex G-25 column (PD-10) were from Pharmacia (USA); ampicillin, bacto-yeast extract and bacto-tryptone were from Oxoid (Cambridge, UK); bacto-agar was from Difco (Beijing, China); agarose was from Promega (Madison, USA); MTT was from Sigma and low molecular standard protein was from Takara (Dalian, China).

1.1.4 Animals

5–6 week-old BALB/c female nude mice (nu/nu), weighing 16–18 g, were obtained from the Vital River Laboratories (Beijing, China).

1.2 Methods

1.2.1 Construction of expression vector pA-scFv-ldp

The scFv-gene-encoding fragment was cloned by polymerase chain reaction from the pAYZ-scFv plasmid DNA using direct primer *p1* (5'-CAGCATATGAACGCGTAC-GCTCAGGTGAAG-3') and reverse primer *p2* (5'-CGCGA-ATTCTGAACCGCCTCCACCTTTGATCTCCACCTTG-GT-3'). The obtained scFv PCR products using *Nde* I, *Mlu* I and *Eco*R I sites were cloned into the 18-T vector, yielding plasmid 18T-scFv. The recombinant 18T-scFv was digested with *Nde* I/*Eco*R I, and the released 760 bp scFv gene fragment was subcloned into the pGEMT-LDP plasmid and digested with *Nde* I/*Eco*R I, creating plasmid pGEMT-scFv-ldp. Then the pGEMT-scFv-ldp vector was digested with *Mlu* I/*Xho* I and the released 1100 bp scFv-ldp fragment was ligated to pAYZ, generating recombinant expression plasmid pAYZ-scFv-ldp. The inserted DNA fragment was confirmed using DNA sequencing.

1.2.2 Expression of fusion protein anti-CD20 scFv-LDP and Western blot analysis

E. coli strain 16C9 transformed with pAYZ-scFv-ldp was cultured in 2×YT medium (bacto-yeast extract 10 g L⁻¹, bacto-tryptone 16 g L⁻¹, NaCl 5 g L⁻¹, ampicillin 50 μg mL⁻¹) at 37°C overnight, whereupon the fermentation broth was centrifuged to collect the bacterial cells. The fusion protein scFv-LDP was induced by resuspending the bacteria in AP5 medium (containing 50 μg mL⁻¹ ampicillin) and incubating, with gentle shaking, for 24 h at 30°C. After induction, four fractions, including medium, periplasmic, soluble cytoplasmic and insoluble samples, were obtained for SDS-PAGE analysis and Western blot detection. For Western blotting, PVDF membranes containing transferred proteins were incubated with a 1:2000 dilution of anti-His-Tag monoclonal antibody for 4–5 h, and the bound antibody was detected using a 1:1500 dilution of goat anti-mouse IgG antibody conjugated with horseradish peroxidase and powerful chemiluminescence detection reagents.

1.2.3 Purification and refolding of fusion protein anti-CD20 scFv-LDP

The bacterial cell pellets were resuspended in binding buffer (5 mmol L⁻¹ imidazole, 0.5 mol L⁻¹ NaCl and 20 mmol L⁻¹ Tris-HCl, pH 7.9), sonicated and the cell lysate centrifuged. This step was then repeated. The resulting pellet was resuspended and incubated in binding buffer containing 6 mol L⁻¹ urea for 1 h. Insoluble material was then removed by centrifugation at 16000 g for 30 min. The supernatant was filtered through a 0.45 μm membrane, and then purified by Ni-NTA resin affinity chromatography column. The resulting purified protein was refolded using stepwise dialysis. Specifically, the anti-CD20 scFv-LDP fusion protein was diluted to 5 μmol L⁻¹ with binding buffer containing 6 mol L⁻¹ urea, and then β-mercaptoethanol was added to a final concentration of 10 mmol L⁻¹. After incubation at room temperature for 30 min, the sample was dialyzed against refolding buffer (50 mmol L⁻¹ Tris-HCl pH 8.0, 1 mmol L⁻¹ EDTA, 200 mmol L⁻¹ NaCl, 6 mol L⁻¹ urea) to remove the β-mercaptoethanol. Dialysis was then subsequently performed against the same buffer with step-wise reduction in the urea concentration (3, 2, 1, 0.5, and 0 mol L⁻¹). At the 1-mol L⁻¹ dialysis, 750 μmol L⁻¹ of glutathione and 400 μmol L⁻¹ of L-arginine were added to the dialysis buffer. The sample was then dialyzed against PBS solution (pH 7.4). After centrifugation at 10000 g for 30 min at 4°C, the supernatant was collected. The protein was condensed by centrifugation at 12000–14000 g and the concentration was determined using a BCA protein assay kit from Pierce.

1.2.4 Enzyme-linked immunosorbent assays (ELISA)

96-well plates were coated with 0.01% polylysine (200 μL well⁻¹) at 4°C overnight and then washed with PBS. Raji or

Daudi cells were grown in 96-well plates, washed with PBS and fixed for 15 min with 0.05% glutaraldehyde/PBS at 4°C. Plates were then washed with PBS, and nonspecific binding was blocked by incubation with 1% BSA/PBS at 4°C overnight. The wells were emptied and washed, 50 μL well⁻¹ of anti-CD20 scFv-LDP was added in two fold serial dilutions at concentrations ranging from 7.50 to 0.06 μmol L⁻¹ and were allowed to stand for 2 h at 37°C. After removing the unbound scFv-LDP, 50 μL well⁻¹ of 1500-fold diluted mouse anti-His-Tag mAb was used as a primary antibody. Following incubation and washing, 50 μL well⁻¹ horseradish peroxidase-conjugated goat anti-mouse IgG, at a dilution of 1:2000, was added as a secondary antibody. A total 100 μL of *o*-phenylenediamine substrate solution was added as the coloring reagent, and the reaction was terminated after 10 min by the addition of 50 μL of 2 mol L⁻¹ H₂SO₄. Absorbance was measured at 492 nm using a microplate reader.

1.2.5 Indirect immunofluorescent cytochemical staining

1×10⁶ Raji cells were resuspended in 30 μL PBS containing 20 μg scFv-LDP and incubated at 4°C for 1 h. The supernatant was removed by centrifugation at 2000 g for 10 min at 4°C. After being washed with PBS, the cells were resuspended in PBS solution containing 1500-fold diluted anti-His-Tag mAb and incubated at 4°C for 1 h. The supernatant was discarded by centrifugation at 2000 g for 10 min at 4°C. The cells were resuspended in 30 μL working solution containing FITC-conjugated goat anti-mouse IgG and incubated at 4°C for 30 min. The supernatant was removed and the cells were fixed in 4% polyformaldehyde. The binding of anti-CD20 scFv-LDP to Raji cells was detected by FACS analysis.

1.2.6 Preparation of energized fusion protein scFv-LDP-AE

10 mg of LDM with high potent activity was suspended in 5 mL methanol and whisked for 5 min at 4°C. The mixture was then placed at -20°C for 1 h. Hence, active enediyne (AE) in the supernatant of the reaction mixture was obtained by centrifugation at 16000 g for 20 min at 4°C. The above procedure was repeated again to isolate AE completely. To obtain energized fusion protein scFv-LDP-AE, AE in methanol was added to scFv-LDP/PBS (10 mmol L⁻¹, pH 7.0) with the molecular ratio of 5:1 and the volume ratio of 1:50. The mixture was then placed at room temperature for 12 h. Purified scFv-LDP-AE was finally separated from free AE using a PD-10 column.

1.2.7 MTT assays

CD20-positive Raji or Daudi cells were seeded at 10000 cells well⁻¹ in 96-well plates, and incubated at 37°C in a humidified 5% CO₂ incubator overnight. Different concentrations of LDM and scFv-LDP-AE were then added and incubated for an additional 72 h. 50 μL of MTT (2 mg mL⁻¹

in serum-free RPMI 1640 medium) was added in each well, and incubated for 4 h at 37°C. The culture supernatant was removed gently, and 150 μ L of DMSO was added to each well. After shaking at room temperature for 15 min, the absorbance was determined using a microplate reader at a wavelength of 570 nm. Based on the average absorbance of triplicate wells, survival ratio was calculated according to the following formula:

$$\text{Survival ratio} = (A_{\text{test}} - A_{\text{blank}}) / (A_{\text{control}} - A_{\text{blank}}) \times 100\%.$$

1.2.8 Animal experiments

Experiments were performed with 5–6 week old female nude mice. At first, they were exposed to total body irradiation (400 rad) to further suppress their residual immune system and facilitate the establishment of xenografts. On the second day, 2×10^7 Raji cells suspended in 0.2 mL PBS were subcutaneously injected into the right flank of the mice. On day 14, the tumors reached approximately 100 mm³. Animals were grouped ($n=6$) and different doses (0.1, 0.2 and 0.3 mg kg⁻¹) of scFv-LDP-AE were administered IV, as was LDM (0.05 mg kg⁻¹) and scFv-LDP (0.3 mg kg⁻¹). Control mice were injected with PBS. On day 21, the drugs being tested were readministered. Tumor size was measured every 3 days and tumor volumes were calculated with the following formula: $V=0.5a \times b^2$, where a and b are the long and the perpendicular short diameters of the tumor, respectively. The data are presented as mean \pm SD. Tumor growth curves were plotted and the inhibitory rates of tumor growth were calculated according to the tumor volume. Student's t -test was used to determine statistically with significant differences. $P < 0.05$ was considered significant.

2 Results

2.1 Construction and expression of the anti-CD20 scFv-LDP fusion protein

In order to create the anti-CD20 scFv-LDP fusion protein, the DNA sequence encoding for the scFv fragment of mAb HI47 was genetically fused to the LDP gene, which encodes for the apoprotein of LDM. Fusion occurred when a five-amino-acid linker (GGGGS) between the C-terminus of scFv and the N-terminus of LDP was cloned into the *Mlu* I/ *Xho* I restriction sites of the pAYZ expression vector, leading to plasmid pAYZ-*scfv-ldp* (Figure 1A). The scFv-LDP fusion gene was under the control of the *phoA* promoter and an (His)₆-Tag was introduced at the C-terminus of the constructs to facilitate the purification via immobilized metal-affinity chromatography. The fusion gene was composed of 1182 bp and encoded for 394 amino acids.

The plasmid vector pAYZ-*scfv-ldp* was transformed into *E. coli* 16C9 and the target protein was induced by AP5 culture medium. As shown in the Coomassie Blue-stained gel in Figure 1B, recombinant proteins accumulated in intracellu-

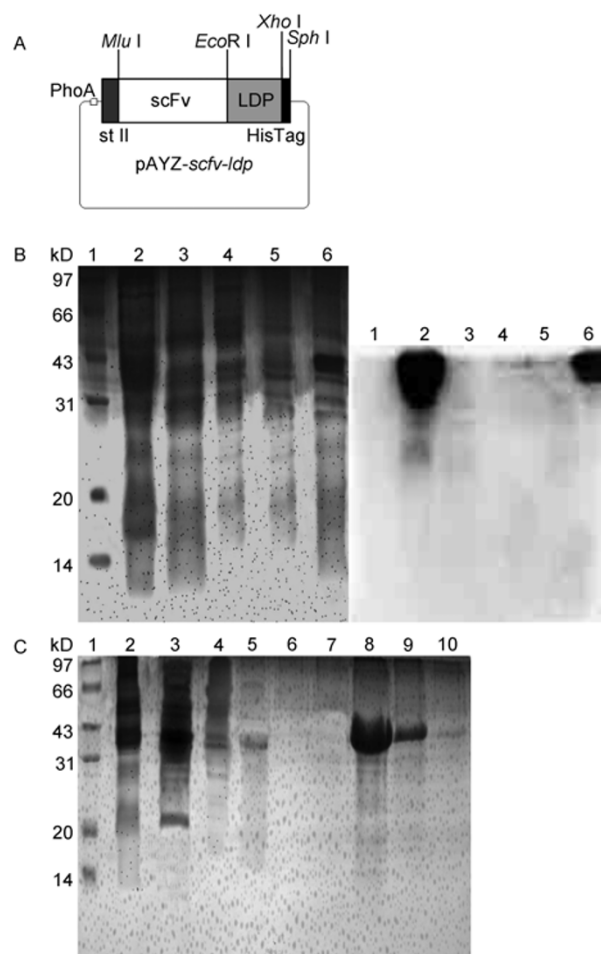


Figure 1 Construction, expression, and purification of the fusion protein, anti-CD20 scFv-LDP. A, Construction of the plasmid pAYZ-*scfv-ldp* carrying the gene for the fusion protein, scFv-LDP. B, SDS-PAGE (left) and western-blot (right) analysis of each fraction of *E. coli* 16C9 cells expressing scFv-LDP fusion proteins. 1: molecular weight marker; 2: total proteins of *E. coli* 16C9 carrying plasmid pAYZ-*scfv-ldp* after induction; 3: medium sample; 4: periplasmic fraction; 5: cytoplasmic soluble fraction; 6: cytoplasmic insoluble fraction. C, Purification of the fusion protein scFv-LDP by Ni-NTA affinity chromatography. 1: molecular weight marker; 2: total cell protein; 3: sample before Ni²⁺ column; 4–7: contaminating protein not bound to the resin; 8–10: scFv-LDP protein obtained by eluting with stripping buffer.

lar inclusion bodies and composed approximately 30% of total cell protein. The proteins were running in good agreement with the theoretical molecular weight of 43 kD for the anti-CD20 scFv-LDP fusion protein. The fusion construct was further confirmed by Western blot using an anti-His-Tag antibody (Figure 1B).

2.2 Purification and refolding of the anti-CD20 scFv-LDP fusion protein

The fusion protein scFv-LDP was purified with Ni-NTA resin under denaturing conditions and target proteins of over 95% purity were obtained, as shown in Figure 1C. After refolding in a stepwise manner (see Materials and methods),

20 mg soluble scFv-LDP could be harvested from 1 L fermentation broth. The protein was further elucidated using SDS-PAGE, and only a single band with a molecular weight of 43 kD could be discerned, indicating that no degradation or aggregation occurred in the process of purification and refolding.

2.3 Binding assays

To confirm the correct folding and functional binding of the fusion protein, the binding ability of anti-CD20 scFv-LDP to CD20-positive tumor cells was examined by ELISA. The data indicated that scFv-LDP bound to the Raji or Daudi cells in a dose-dependent and saturable manner. scFv-LDP interacted with Raji and Daudi cells with relative affinities of 4×10^{-7} and 8×10^{-7} mol L⁻¹, respectively, which is slightly lower than that of corresponding scFv (Figure 2A and B). Indirect immunofluorescent cytochemical staining and FACS analysis also showed that both scFv and scFv-LDP could specifically bind to Raji or Daudi cells, and the binding activity of scFv was slightly higher than that of scFv-LDP (Figure 2C). The results indicated that anti-CD20 scFv-LDP retained part of the antigen-binding affinity of the parent antibody.

2.4 Generation of the energized fusion protein anti-CD20 scFv-LDP-AE

By adding AE, the active enediyne chromophore of LDM, to anti-CD20 scFv-LDP fusion protein solution in controlled conditions, the energized fusion protein scFv-LDP-AE was prepared. scFv-LDP-AE was purified from free AE after separating on a PD-10 column. We found that the scFv-

LDP-AE molecules showed strong absorbance at both 343 and 280 nm, representing the chromophore and protein moiety, respectively (Figure 3).

2.5 Death of lymphoma cells *in vitro* due to the energized fusion protein scFv-LDP-AE

Cytotoxicity of scFv-LDP-AE and LDM to tumor cells was determined by MTT assay (Figure 4). Both scFv-LDP-AE and LDM displayed extremely potent cytotoxicity to different tumor cells. The IC₅₀ values of scFv-LDP-AE and LDM in CD20-positive Raji cells were 1.21×10^{-11} and 7.13×10^{-11} mol L⁻¹, respectively, and 6.24×10^{-11} and 2.91×10^{-10} mol L⁻¹ in CD20-positive Daudi cells. The IC₅₀ values of scFv-LDP-AE and LDM in CD20-negative MCF7 cells were 3.39×10^{-9} and 5.91×10^{-10} mol L⁻¹, respectively. The results suggested that the scFv-LDP-AE showed greater cytotoxicity in CD20-positive lymphoma cells than free LDM, which was most likely due to the targeting effects of anti-CD20 scFv in the fusion construct.

2.6 Animal experiments

Antitumor experiments were performed with subcutaneous Raji xenografts established in nude mice. As shown in Figure 5, both scFv-LDP-AE and LDM could inhibit or retard the growth of Raji xenografts significantly, with scFv-LDP-AE inhibiting tumor growth in a dose-dependent manner. As evaluated on day 41, scFv-LDP-AE at 0.2 and 0.3 mg kg⁻¹ suppressed the tumor growth by 70.5% and 79.3%, respectively, whereas free LDM at the tolerated dose of 0.05 mg kg⁻¹ showed an inhibition rate of 68.6%. The inhibition rate of scFv-LDP-AE at 0.3 mg kg⁻¹ demonstrated

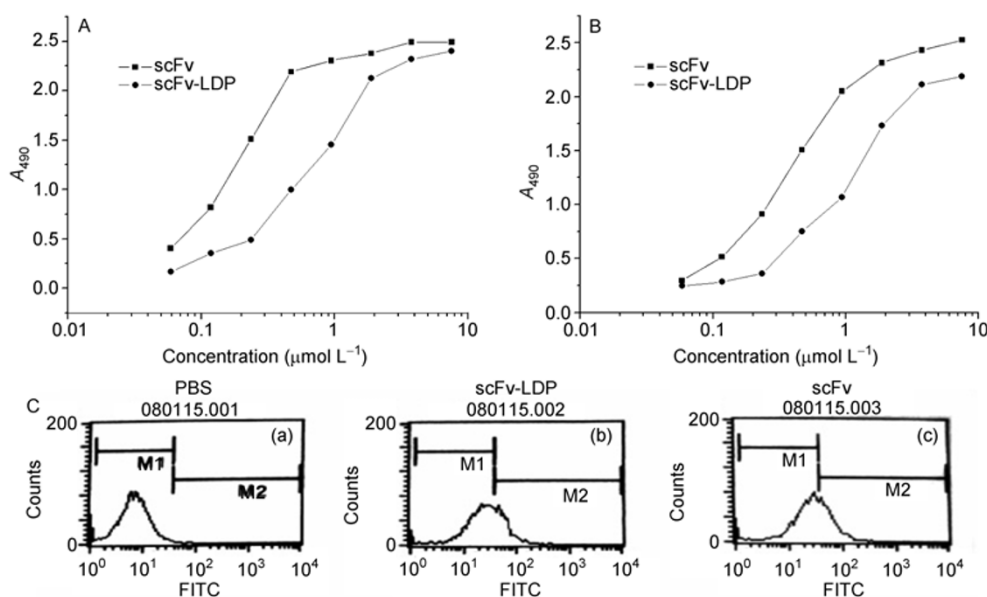


Figure 2 Immunoreactivity analysis of anti-CD20 scFv and scFv-LDP. A, Immunoreactivity to Raji and Daudi cells by ELISA; B, immunoreactivity to Daudi cells by ELISA; C, specific binding to Raji cells by indirect immunofluorescence and FACS analysis.

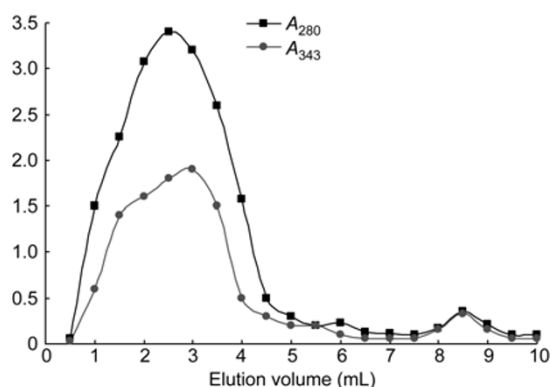


Figure 3 Separation of the energized fusion protein anti-CD20 scFv-LDP-AE from free AE on a PD-10 column (Sephadex G-25; Amersham). Elute, 1×PBS.

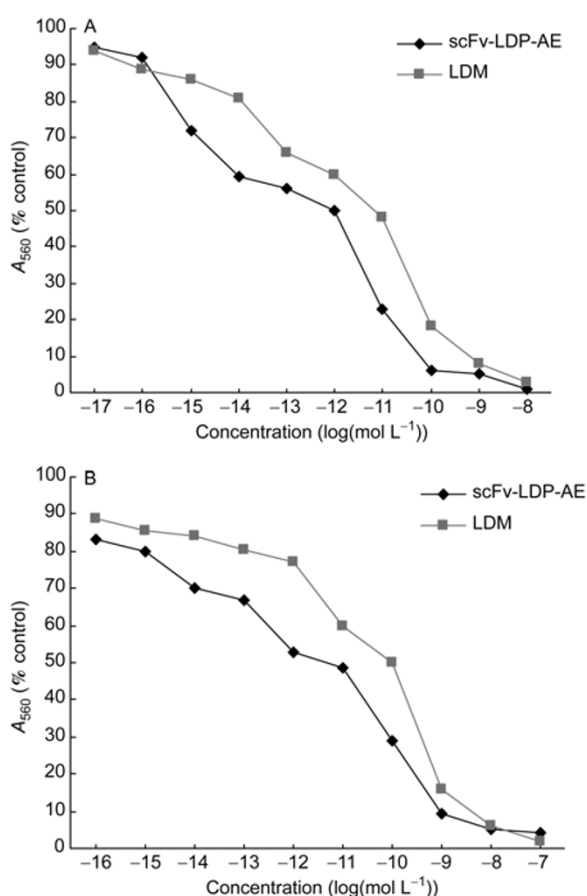


Figure 4 Growth inhibition effects of scFv-LDP-AE and LDM on Raji (A) and Daudi (B) cells determined using MTT assay.

statistically significant differences ($P < 0.05$) compared with that of LDM at 0.05 mg kg^{-1} . Almost no inhibitory effect could be achieved in the group treated with fusion protein scFv-LDP at 0.3 mg kg^{-1} compared with the control group. All treated mice survived at that time of the study. No severe side-effects were observed during the treatment, which implied that the therapeutic efficacy of scFv-LDP-AE at a tolerated dose was stronger than that of free LDM.

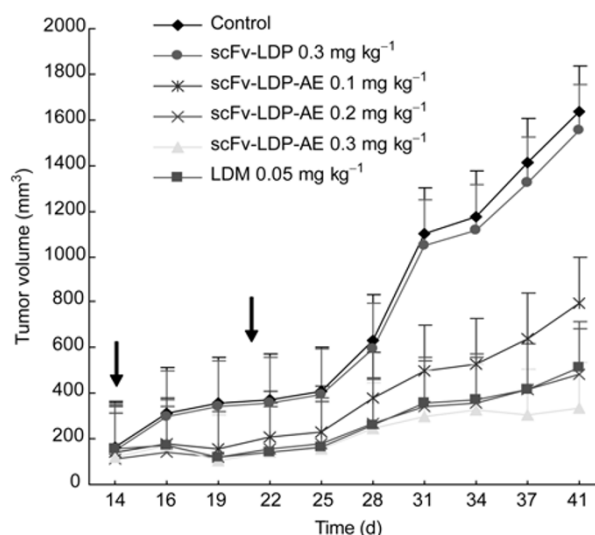


Figure 5 Energized fusion protein scFv-LDP-AE inhibited the growth of Raji xenografts in nude mice.

3 Discussion

Since Rituximab (RTX), the CD20-specific chimeric monoclonal antibody, was approved by the US FDA in 1997 for B cell NHL, markedly improved clinical responses in B-lymphoid malignancies have been observed [23]. The success of RTX presents clear opportunity for antibody-based agents in lymphoma therapy. Unfortunately, approximately 50% of patients with aggressive B-cell NHL are primary refractory or develop relapses after treatment with RTX. The development of Y-90 ibritumomab tiuxetan (Zevalin) and I-131 tositumomab (Bexxar), radiolabelled antibodies with a different mechanism of action compared to RTX, overcame the problem of RTX-resistance. These compounds, however, have their own problems, such as a low response rate following the second treatment, short duration of response, a higher risk for secondary tumors, serious side effects, and poor tolerance in patients [24,25]. Therefore, improvements in this area are still greatly required.

Engineered fusion proteins, composed of an antibody fragment and an effector molecule, represent a new class of highly active antibody-based therapeutic that is now being developed for immunotherapy of malignant tumors. In this study we explored a new antibody-targeting fusion protein anti-CD20 scFv-LDP-AE. Anti-CD20 scFv-LDP-AE is composed of an anti-CD20 scFv fragment and LDM. The scFv moiety plays a crucial role in transferring the LDM to the tumor site, and then LDM kills the tumor cells by causing double strand DNA cleavage [26,27]. The fusion construct, therefore, allows the toxic side effects of LDM in normal cells to be avoided. Our results indicate that the engineered and energized fusion protein scFv-LDP-AE not only showed potent cytotoxicity to cancer cells, but also demonstrated significant antitumor effects during *in vivo* experiments. The fusion protein (0.3 mg kg^{-1}) suppressed

the growth of Raji xenografts by 79.3%, which demonstrated statistically significant differences ($P < 0.05$) compared with that of LDM at the maximal tolerated doses of 0.05 mg kg^{-1} . This finding suggests that scFv-LDP-AE increases the therapeutic potential of LDM.

scFv-LDP-AE and Rituximab are antibody-based drugs for the same target, CD20, and used in the therapy of B-cell NHL. However, the therapeutic effect of RTX depends on the host immune effector system, including antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Continuous administration of RTX and high tumor burden can exhaust host effector function and nullify the effect of RTX [28]. The radiolabelled antibodies, Zevalin and Bexxar, exert effects dependent on β -emissions with path lengths of 1–5 mm, and thus avoid the effector exhaustion problem of RTX. γ -radiation, however, is a safety concern as they can damage nearby progenitor cells in bone marrow, and cause pancytopenia. Sublethal radiation damage to bone marrow stem cells increases the risk of myelodysplastic syndrome and/or acute myelogenous leukemia. Interestingly, anti-CD20 scFv-LDP-AE elicits cytotoxicity through conjugated LDM without any γ -radiation, rather than ADCC or CDC effects, and therefore avoids the effector-exhaustion problem, which hampered the efficacy of RTX [29]. In addition, the hypoxic nature of cells within solid tumors limits the efficacy of anticancer therapies, such as ionizing radiation and conventional radiomimetics, because their mechanisms of action require oxygen to induce lethal DNA breaks. Terry *et al.* [30] reported that LDM could preferentially target hypoxic cells within tumors and overcome the radioresistance associated with poorly oxygenated cells. Therefore, anti-CD20 scFv-LDP-AE has the potential to overcome both the RTX-resistance and the radioresistance associated with poorly oxygenated cells in the treatment of B-cell NHL.

Because the LDM molecule can be separated and reconstituted, we prepared the engineered and energized fusion protein anti-CD20 scFv-LDP-AE consisting of an anti-CD20 scFv fragment and LDM using DNA recombination and molecular reconstitution. In contrast to traditional chemical coupling approaches, which lead to heterogeneous products and a complex manufacturing process, our methods preserve the high-order structure and activity of the antibody moiety, and easily obtained the perfect 1:1 antibody-effector ratio in the final products. Furthermore, we can obtain the target protein simply in *E. coli* and avoid the complex and expensive mammalian cell expression systems commonly used for the expression of recombinant antibodies at present time. Therefore, the fusion protein anti-CD20 scFv-LDP-AE could be the next generation of antibody-based drugs offering the potential to overcome RTX-resistance and radioresistance and may be a promising therapy against CD20-positive B-cell lymphoma.

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